

Functional Expression and Stabilization of Horseradish Peroxidase by Directed Evolution in *Saccharomyces cerevisiae*

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Abstract: Biotechnology applications of horseradish peroxidase (HRP) would benefit from access to tailor-made variants with greater specific activity, lower K_m for peroxide, and higher thermostability. Starting with a mutant that is functionally expressed in *Saccharomyces cerevisiae*, we used random mutagenesis, recombination, and screening to identify HRP-C mutants that are more active and stable to incubation in hydrogen peroxide at 50°C. A single mutation (N175S) in the HRP active site was found to improve thermal stability. Introducing this mutation into an HRP variant evolved for higher activity yielded HRP 13A7-N175S, whose half-life at 60°C and pH 7.0 is three times that of wild-type (recombinant) HRP and a commercially available HRP preparation from Sigma (St. Louis, MO). The variant is also more stable in the presence of H₂O₂, SDS, salts (NaCl and urea), and at different pH values. Furthermore, this variant is more active towards a variety of small organic substrates frequently used in diagnostic applications. Site-directed mutagenesis to replace each of the four methionine residues in HRP (M83, M181, M281, M284) with isoleucine revealed no mutation that significantly increased the enzyme's stability to hydrogen peroxide. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 76: 99–107, 2001.

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INTRODUCTION

Horseradish peroxidase (HRP), an extracellular heme enzyme isolated from horseradish roots, oxidizes a variety of primarily aromatic substrates using hydrogen peroxide as oxidant (Ryan et al., 1994). The most abundant of the more than 40 HRP isoenzymes (Dunford, 1991), HRP-C consists of 308 amino acids and a single protoporphyrin IX prosthetic group, two calcium ions, and four disulfide bridges (Morishima et al., 1986; Haschke and Friedhoff, 1978). This basic isoenzyme has eight N-linked carbohydrate chains which account for 18–22% of its molecular weight (Welinder, 1979). The HRP catalytic cycle consists of a single two-electron transfer (H₂O₂ is the oxidizing substrate) to

form an enzyme intermediate known as Compound I. Two single-electron transfers from substrate molecules (mostly small aromatic molecules) reduce Compound I to Compound II and then to the native resting state. In the case of phenols and anilines the substrate molecules providing the electrons diffuse into the solvent as free radicals and polymerize (Dunford, 1991).

HRP has long been utilized in life sciences as a reporter for a variety of diagnostic assays, in histochemical staining, and in biosensors (Veitch and Smith, 2000). HRP has been considered for applications in chemical synthesis, where it catalyzes a number of potentially interesting oxidative reactions, including dehydrogenation and polymerization of aromatic compounds, heteroatom oxidations, and epoxidation (Colonna et al., 1999; Veitch and Smith, 2000). HRP applications as an indicator for food processing (Weng et al., 1991) and for removal of phenols and aromatic amines from waste waters (Nicell et al., 1993) have also been proposed. These diverse possibilities reflect the broad substrate specificity and high specific activity of the natural enzyme. However, actual commercial uses have been limited to the diagnostic arena due to a number of factors. One is the enzyme's limited stability under the prevalent conditions, including elevated temperatures and in the presence of hydrogen peroxide.

We are using methods of directed evolution (Morawski et al., 2000; Cherry et al., 1999; Petrounia and Arnold, 2000) to engineer new catalysts starting from HRP-C. Stability, activity towards nonnatural substrates, and expression in heterologous hosts are all important features that one should be able to manipulate using these methods. Directed evolution, however, requires functional expression in a host that is suitable for making and screening libraries of thousands, and preferably tens of thousands, of mutants. HRP is expressed in *E. coli* in inclusion bodies (Smith et al., 1990) and functionally in yeast (*S. cerevisiae*) only at very low levels (30 µg/L) (Vlami-Gardikas et al., 1992), too low to conveniently screen for interesting mutants. In previous work (Morawski et al., 2000), we used random mutagenesis, recombination, and screening to identify HRP mutants that are expressed at higher levels in *S. cerevisiae* and that also have higher specific activity than the wildtype enzyme. By

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Abbreviations: HRP—horseradish peroxidase, TMB—tetramethylbenzidine, OPD—*o*-phenylenediamine, ABTS—2,2'-azino-bis-ethylbenzthiazoline-6-sulfonate, ODI—*o*-dianisidine.

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expressing those mutants in *Pichia pastoris*, we could obtain quantities sufficient for purification and further characterization. Here we describe the further evolution of HRP to make enzymes that are more thermostable, more highly active towards an array of HRP substrates, and whose K_m for peroxide is reduced.

MATERIALS AND METHODS

Materials

All chemicals were of reagent-grade purity. ABTS (2,2'-azino-bis-ethylbenzthiazoline-6-sulfonate), tetramethylbenzidine (TMB), guaiacol, 5-aminosalicylic acid (5AS), *o*-phenyldiamine (OPD), *o*-dianisidine (ODI), and H₂O₂ were from Sigma (St. Louis, MO). Restriction enzymes and ligase were from New England Biolabs (Beverly, MA). *Taq* DNA polymerases were from Boehringer Mannheim (Indianapolis, IN). Proofreading polymerase *Pfu* and *E. coli* XL1-Blue supercompetent cells were from Stratagene (La Jolla, CA). dNTPs were purchased from Boehringer-Mannheim. Plasmid pYEX-S1 was obtained from Clontech (Palo Alto, CA). *Saccharomyces cerevisiae* strain BJ5465 (*ade2-1*, *ura3-52*, *trp3-11*, *pep-his*, *poblΔ1*, *6R*, *can1-100*, *GAL*) was obtained from the Yeast Genetic Stock Center (University of California, Berkeley). *Escherichia coli* HB101 electro-competent cells, *Pichia pastoris* strains X-33, and plasmid pPIZαB were obtained from Invitrogen (Carlsbad, CA). The Gietz lab yeast transformation kit was from Tetra-Link (Amherst, NY). The yeast plasmid miniprep kit was from Zymo Research (Orange, CA).

Mutagenesis and Recombination

Error-Prone PCR

Libraries of HRP mutants were constructed by error-prone PCR essentially as described previously (Morawski et al., 2000). In short, 30 pmole of each primer flanking the HRP gene (5'-CAG TTA ACC CCT ACA TTC-3' and 5'-TGA TGC TGT CGC CGA AGA AG-3'), 5 mM MgCl₂, 0.2 mM of each dATP and dGTP, 1 mM of each dTTP and dCTP were used in the PCR reactions. Thermal cycling parameters were 94°C for 3 min (1 cycle), 94°C for 1 min, 48°C for 1 min, 72°C for 1.5 min (30 cycles), and 72°C for 10 min (1 cycle). The PCR products were purified and digested with *Sac* I and *Bam* HI (leaving the first 27 amino acid residues of HRP unmodified). The digestion products were gel-purified and the HRP fragments were ligated into the similarly digested vector pYEXS1-HRP. Ligation mixtures were transformed into *E. coli* HB101 cells by electroporation with a Gene Pulser II from Bio-Rad (Hercules, CA) and selected on LB medium supplemented with 100 μg/ml ampicillin. Colonies were directly harvested from LB plates, mixed, and plasmid DNA isolated. This plasmid DNA was used for transformation into protease-deficient *S. cerevisiae*

BJ 5465. Yeast transformation was carried out with a modified LiAc method as described previously (Gietz et al., 1995), followed by plating on YNB-selective medium (0.67% yeast nitrogen base without amino acids, 20 μg/ml leucine, 20 μg/ml histidine, 40 μg/ml adenine, 20 μg/ml tryptophan) and incubation at 30°C for 48–60 h to recover transformants.

StEP Recombination

Ten ng of each DNA template (plasmids of one high-activity mutant HRP H1-6E1, two stability mutants HRP H2-10G5, HRP H2-10C5, and wild-type) were mixed with 30 pmole of each primer (forward 5'-GGT AAG GCC ATT GAA GAT GC-3' and reverse 5'-GCT GTC ATT CGT TCA AGG TTT GG-3'), 0.2 mM dNTPs, 2.5 Units of *Taq* polymerase, and *Taq* polymerase buffer. PCR conditions were 1 cycle at 94°C for 2 min, 100 cycles of 94°C for 30 sec, 55°C for 25 sec. The resulting reaction mixture was treated with *DpnI* and the gene reamplified using standard PCR conditions with nested forward primer 5'-CAG TTA ACC CCT ACA TTC-3' and reverse primer 5'-TGA TGC TGT CGC CGA AGA AG-3'. The resulting PCR product was digested and ligated into vector pYEXS1.

Saturation Mutagenesis

Fifty ng of DNA template (pYEXS1 plasmid containing HRP 13A7) was mixed with 30 pmole of forward primer 5'-CAC ACA TTT GGA AAG NNN CAG TGT AGG TTC ATC-3' and reverse primer 5'-GAT GAA CCT ACA CTG NNN CTT TCC AAA TGT GTG-3' (site of variable nucleotides is underlined), 0.2 mM dNTPs, 2.5U *Pfu* polymerase, and *Pfu* polymerase buffer (total volume 50 μl). PCR conditions were 1 cycle at 95°C for 2 min, 16 cycles of 95°C for 30 sec, 55°C for 1 min, and 68°C for 20 min. The resulting reaction mixture was treated with *DpnI* and transformed in *E. coli* XL-1 Blue cells. Plasmid DNA was then isolated and transformed into *S. cerevisiae*.

Site-Directed Mutagenesis

Conditions used were essentially the same as for saturation mutagenesis. Mutations M83I, M181I, M281I, M284I, and M281-284I were introduced into HRP 13A7 and HRP H2-10G5 using appropriate primers. Mutation N175S was introduced into HRP-C wild-type using forward primer 5'-CAC ACA TTT GGA AAG AGC CAG TGT AGG TTC ATC-3' and reverse primer 5'-GAT GAA CCT ACA CTG GCT CTT TCC AAA TGT GTG-3' (underlined nucleotides encode serine).

Screening HRP Mutant Libraries

Single colonies were picked from plates containing selective YNB medium and grown at 30°C for 64 h in 96-well plates containing nonselective YEPD medium (1% yeast

extract, 1% peptone, 2% glucose). Then 100 μ l of the supernatant in each well was transferred to a V-bottom microplate (MJ Research, Waltham, MA) with a Beckman 96-channel pipetting station (Multimek; Beckman, Fullerton, CA). Each well contained 100 μ l NaOAc buffer (50 mM, pH 4.5) with 0.5–1 mM H₂O₂. Forty μ l were transferred to wells of a new 96-well plate and assayed for total initial HRP activity. After incubation of the V-bottom microplate at 50°C for 10 min, another 40 μ l were transferred to another 96-well plate and assayed for residual activity. Stability is indicated by the ratio of the residual to initial activity.

Activity assays were performed in NaOAc buffer (50 mM, pH 4.5) containing 2 mM ABTS and 1 mM H₂O₂. Absorbance at 405 nm ($\epsilon = 34,700 \text{ cm}^{-1}\text{M}^{-1}$ for oxidized ABTS) was monitored using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 25°C. Standard deviations for this measurement in 96-well plates containing parent clones were 14–17%.

Mutants showing the highest total HRP activity and stability were retrieved from the microtiter plates and re-screened after growth in 5-ml culture of YEPD medium. Thermostability was probed by incubating a 1:1 mixture of 100 μ l supernatant and 100 μ l NaOAc buffer (50 mM, pH 4.5) at various temperatures for 10 min. Initial and residual activities were determined again in NaOAc buffer (50 mM, pH 4.5) with H₂O₂ (1 mM) and ABTS (2 mM). Tests for H₂O₂ stability and thermostability with purified protein samples were performed in the same way, using 75 ng/ml of protein in the incubation samples. Plasmids were extracted from the cells with a Zymo yeast plasmid miniprep kit and returned to *E. coli* JM109 for further propagation and comparative isolation.

HRP Activity Assays on Enzyme Purified from *Pichia pastoris*

All assays were performed at 25°C. Kinetics for ABTS oxidation were determined using 3–11 ng of purified enzyme, 3 mM H₂O₂ and 0–1.8 mM ABTS in NaOAc buffer (50 mM, pH 4.5). Kinetics for H₂O₂ reduction were determined with 3–11 ng of purified enzyme, 3 mM ABTS, and 0–0.1 mM H₂O₂.

Kinetics for guaiacol oxidation were performed with 6–23 ng of protein, 3 mM H₂O₂, 0–5 mM guaiacol in sodium phosphate buffer (50 mM, pH 7.0). The increase in absorbance at 470 nm was followed (ϵ_{470} of oxidized product = $26,000 \text{ cm}^{-1}\text{M}^{-1}$) after addition of the purified protein.

Tetramethylbenzidine (TMB) assays were performed with 0.5–2.3 ng of protein, 2 mM H₂O₂, and 4 mM TMB (50 mM stock solution in H₂O with 20% ethanol) in water. The increase in absorbance at 650 nm was followed (blue color development, $\epsilon_{650} = 39,000 \text{ cm}^{-1}\text{M}^{-1}$).

5-Aminosalicylic acid (5AS) and *o*-phenyldiamine (OPD) assays were performed using 10–23 ng of protein, 1 mM H₂O₂, and 5 mM 5AS or 5 mM OPD in sodium phos-

phate buffer (50 mM, pH 7.0). The increase in absorbance was measured at 470 nm for 5AS and 405 nm for OPD.

The *o*-dianisidine (ODI) assay was performed with 5–11 ng of protein, 1 mM H₂O₂ and 2 mM ODI in water. Increase in absorbance was followed at 505 nm.

Vector Construction for HRP Expression in *Pichia pastoris*

Mutated HRP genes were cloned into *Pichia* expression vector pPIZ α B containing the α -factor secretion signal and the methanol-inducible P_{AOX1} promoter. pPIZ α B containing HRP wild-type (Morawski et al., 2000) was digested with *Hpa*I and *Eco*RI and purified. The coding sequences for the HRP variants were obtained from the corresponding pYEXS1-HRP plasmids by PCR techniques using *Pfu* polymerase and two primers, 5'-AAC CCC TAC ATT CTA CGA CAA TAG CT GTC CC-3' (forward) and 5'-CCA CCA CCA GTA GAG ACA TGG-3' (reverse). The PCR products were digested with *Eco*RI and ligated into pPIZ α B, transformed into *E. coli* Top10F' and selected on low salt LB media (1% tryptophan, 0.5% yeast extract, 0.5% NaCl, pH adjusted to 7.5) supplemented with 25 μ g/ml Zeocin. The plasmids containing the HRP genes, as determined by colony PCR, were isolated and confirmed by sequencing. Plasmids were isolated and transformed into *Pichia* X-33 by electroporation according to the supplier's instructions (Invitrogen).

HRP Expression in *Pichia pastoris*

Pichia cells were grown at 30°C in shake flasks. pPIZ α B-HRP-harboring cells were first grown overnight to an OD₆₀₀ of 1.21–1.6 in BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol) supplemented with 1% casamino acids. The cells were pelleted and resuspended to an OD₆₀₀ of 1.0 in BMMY medium (identical to BMGY except 0.5% methanol in lieu of 1% glycerol) supplemented with 1% casamino acids. Growth was continued for another 72–150 h. Sterile methanol was added every 24 h to maintain induction conditions. HRP levels in the supernatants peaked around 80–90 h postinduction (at which time the OD₆₀₀ reached 8.0–10.0).

Purification of HRPs Expressed in *Pichia pastoris*

Yeast cultures were harvested after 87 h of growth and centrifuged at 5,000 rpm on a GS-3 rotor for 20 min. 60% (NH₄)₂SO₄ was added to the supernatant, which was loaded on a phenylsepharose 6 Fast Flow column (Pharmacia) equilibrated with 60% ammonium sulfate in 50 mM phosphate buffer pH 7.0, flow rate 1.5 ml/min. Protein was eluted in a 60% to 0% (NH₄)₂SO₄ gradient. Fractions with the highest ABTS activity were combined and concentrated with an Amicon membrane filtration device (Millipore Corp., Bedford, MA). The protein sample was then loaded

on a 16 × 60 mm Sephacryl S-200 (Pharmacia) column equilibrated with 50 mM phosphate buffer pH 7.0 and 50 mM KCl and eluted in the same buffer. Flow rate was 0.25 ml/min. Fractions with highest peroxidase activity were pooled, dialyzed against 50 mM phosphate buffer pH 7.0, and applied to a MonoQ (Pharmacia) column and eluted in a 0–1 M NaCl gradient in 50 mM phosphate buffer (pH 7.0) to remove impurities.

Total protein concentration was determined after Bradford (1978) with BSA as standard. RZ values (A_{404}/A_{280}) (Dunford, 1991) were between 0.4–0.6 for the final protein samples.

RESULTS AND DISCUSSION

Directed Evolution of HRP

Error-Prone PCR

Since total activity of wild-type HRP-C in *S. cerevisiae* is near the detection limit for ABTS as substrate, a much more highly expressed HRP mutant, 2-13A10 (Morawski et al., 2000) was used as the starting point for directed evolution by random mutagenesis. HRP 2-13A10 is less thermostable than wild-type (residual/initial activity is 2-fold lower after incubation of *S. cerevisiae* supernatant at 65°C). This mutant contains five amino acid substitutions and two synonymous mutations.

Different PCR conditions (0.15 mM MnCl₂ and 7 mM MgCl₂, 0.10 mM MnCl₂ and 7 mM MgCl₂, 7 mM MgCl₂, 5 mM MgCl₂ and otherwise as described in Materials and Methods) producing between 31–71% inactive clones were tested. Mutant libraries were screened for activity and their ability to survive incubation at 50°C in 0.5 mM H₂O₂ (see Materials and Methods). Libraries with a high mutation rate (>50% inactive clones) produced no mutants with increased activity or stability against H₂O₂ and elevated temperature (3,300 colonies screened). Screening lower error-rate libraries produced several mutants with higher total activity (1.45–1.8-fold). Two of those (HRP H1-8H10, HRP H1-6E1) also retained the stability of the parent (Fig. 1). Further screening (a total of 8,000 colonies were screened in this library) revealed no other stable mutants.

A second round of error-prone PCR was applied using HRP H1-8H10 as template. PCR conditions giving 30% inactive clones and screening 3,000 colonies yielded two mutants (HRP H2-10G5 and HRP H2-10C5) with increased stability, but decreased total activity towards ABTS (Fig. 1).

The thermostability of this mutant was investigated at different temperatures (no peroxide during incubation). As shown in Figure 2, mutant HRP H2-10G5 retained 64% of its activity after 10 min incubation of the supernatant at 70°C, whereas the parent lost all activity and wild-type retained only 34% activity under these conditions. H2-10G5 contains one new mutation with respect to HRP H1-8H10, leading to amino acid substitution N175S. Mutant HRP H2-

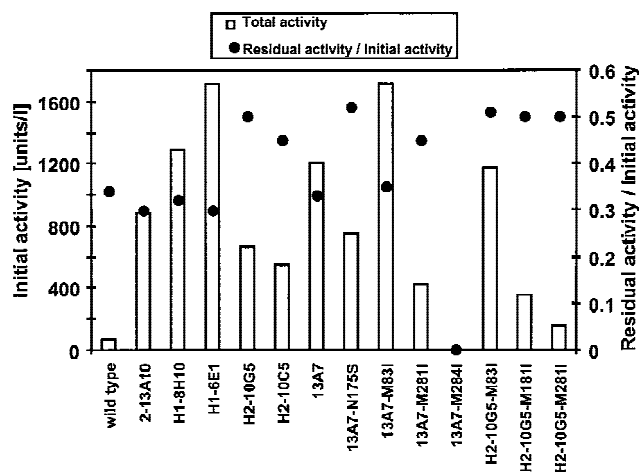


Figure 1. Total activities and stabilities of wild-type and evolved HRP variants expressed in *S. cerevisiae* BJ5465. Activities (determined with the ABTS assay as described in Materials and Methods) are not normalized with respect to cell density (OD_{600}), since little variation in cell density was observed. Cells were grown in 5 ml cultures at 30°C for 64 h. Residual activities measured after 10-min incubation at 50°C in NaOAc buffer (50 mM, pH 4.5) containing 0.5 mM H₂O₂. Stability was determined from the ratio of residual to initial activity.

10C5 also contains one new mutation (F221I). Figure 3 summarizes the mutations introduced.

StEP Recombination

The StEP method (Zhao et al., 1998) was used to recombine wild-type HRP-C, HRP H1-6E1 (first generation), HRP H2-10G5, and HRP H2-10C5 (second generation) (Fig. 3) in order to generate mutants that are both highly stable and active. Sequencing four mutants showed that recombination was successful, i.e., one or two amino acids were exchanged between parent templates in each gene. From this library 1,400 colonies were screened but no mutant that was both

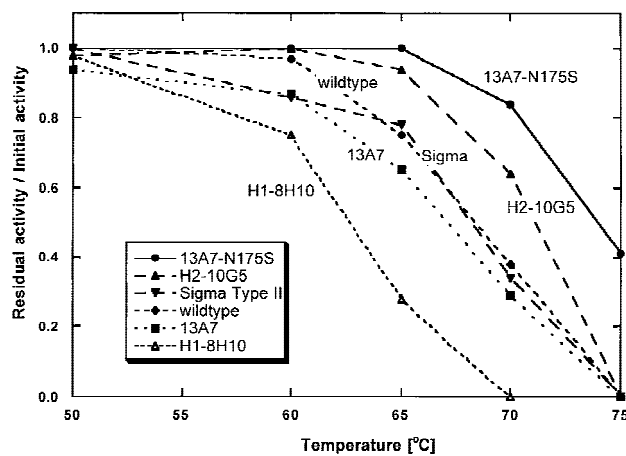


Figure 2. Evolution of thermal stability. Yeast culture supernatants expressing HRP wild-type and isolated HRP variants were assayed for thermal stability (residual activity/initial activity) after incubation for 10 min in NaOAc buffer (50 mM, pH 4.5) at a temperature range between 50–75°C.

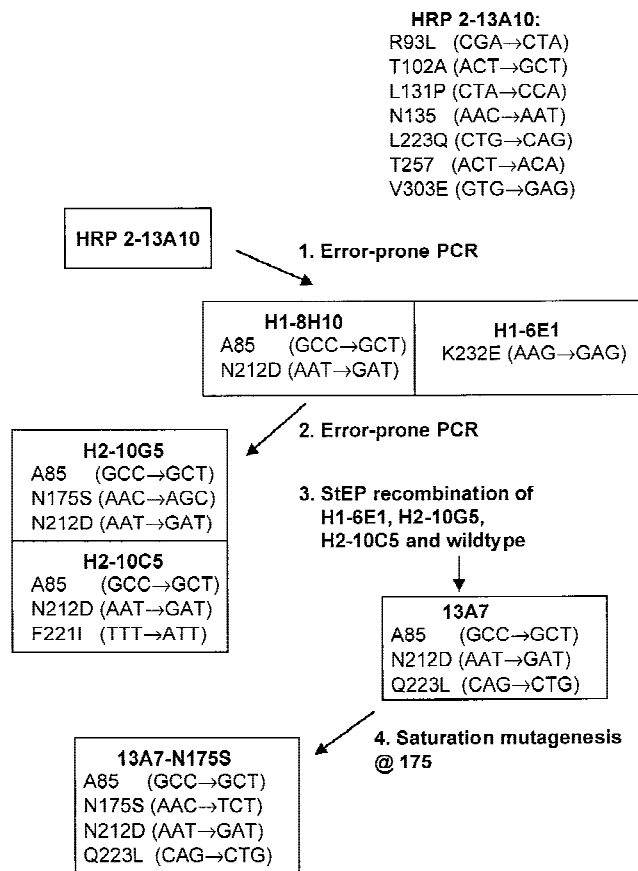


Figure 3. Synonymous and nonsynonymous mutations in isolated HRP variants. Parent HRP 2-13A10 contains a total of five amino acid substitutions and two synonymous mutations. These mutations are retained during the evolution process. Only newly introduced mutations are shown.

more stable and more active than wild-type could be detected. One variant (HRP 13A7) showed high activity accompanied by a return to wild-type thermostability, but no increase in stability in the presence of H₂O₂ at 50°C (Figs. 1, 2). Thermostability, which had decreased in the parents used as templates for the first and second generations, was restored in HRP 13A7 by reversion back to wild-type of a mutation (Q223L) introduced in the earlier expression experiments (Morawski et al., 2000).

Saturation Mutagenesis

To explore other possible thermostabilizing mutations that might not be accessible by point mutagenesis (Miyazaki et al., 1999), we applied saturation mutagenesis at position N175 of thermostable mutant 13A7. Most (94%) of the screened colonies showed decreased total activity. Two mutants with the highest stability were selected and their thermostabilities were determined (Fig. 2). Both were significantly more thermostable even than H2-10G5 (Fig. 2). Both contained a single mutation leading to substitution of Asn to Ser at position 175 (13A7-N175S). Thus, saturation mutagenesis revealed no amino acid substitution more effective

than Ser at this position. The evolution of HRP activity and stability is summarized in Figure 4.

Site-Directed Mutagenesis

To determine if the N175S substitution alone increases thermostability, this mutation was introduced into wild-type HRP. Because N175S introduced in wild-type decreased the total activity in the *S. cerevisiae* supernatant by ~50%, we had to concentrate the supernatant (8-fold) using spin columns (Amicon, Beverly, MA) to test activity and stability. After incubation at 70°C the residual to initial activity ratio was 2-fold higher than wild-type; it was 8-fold higher at 75°C. Wild-type HRP also shows residual activity after incubation at 75°C when similarly concentrated, indicating that the rich medium or the other secreted proteins exert a stabilizing effect. This effect is also seen when the stabilities of the variants measured in supernatant are compared to those determined with purified enzymes in buffer (Table I).

In an independent, rational approach, we investigated how the methionine residues might contribute to the degradation of HRP by H₂O₂. The oxidation of methionine residues is responsible for loss of biological activity in a number of proteins (Volkin et al., 1995). We used site-directed mutagenesis to replace the four methionine residues (M83, M181, M281, M284) with isoleucine, which has similar characteristics of size and hydrophobicity. Mutations were introduced into HRP 13A7 and HRP H2-10G5 and the mutants were expressed in *S. cerevisiae*. The results are summarized in Figure 1. None of the site-directed mutants exhibited dramatically increased H₂O₂ stability compared to the parents. A slight increase (15%) could be seen in the M281I variant. Interestingly, M83I increased the total activity, whereas M181I and M281I decreased activity. M284I inactivated HRP completely, as did the double mutation M281I-M284I.

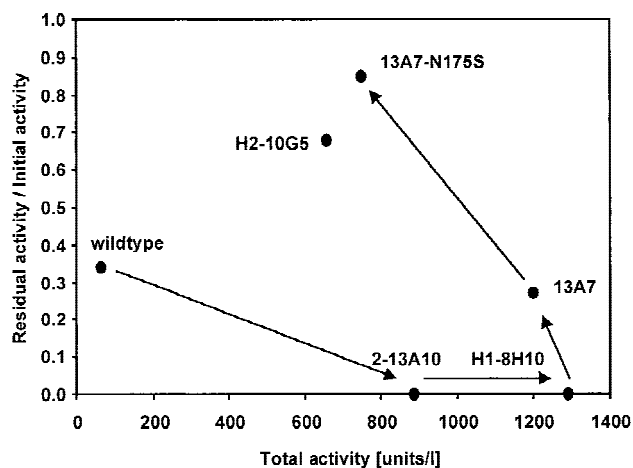


Figure 4. Evolution of the total activity and thermal stability of HRP at 70°C (measured in *S. cerevisiae* supernatant as described in Materials and Methods).

Table I. Stabilities to different conditions of HRP-C Type II (Sigma) and HRP-C wild-type and variants expressed in *Pichia pastoris*

Enzyme	pH 4.5 (60°C)	pH 7.0 (60°C)	pH 8.5 (60°C)	1 mM H ₂ O ₂ (37°C, pH 4.5)	1 mM H ₂ O ₂ (50°C, pH 4.5)	0.5% SDS (50°C, pH 4.5)	8 M urea (60°C, pH 8.5)	3 M NaCl (60°C, pH 7.0)
HRP Type II (Sigma)	0.15	0.1	0	0.42	0.32	0	0.14	0.47
HRP wild-type	0.14	0.1	0	0.56	0.33	0.14	0.23	0.47
HRP 13A7	0.05	0	0	0.52	0.29	0.15	0	0.33
HRP H2-10G5	0.39	0.35	0.13	0.45	0.48	0.27	0.37	1.0
HRP 13A7-N175S	0.55	0.50	0.43	0.50	0.52	0.44	0.67	0.97

Residual activity was measured after 10-min incubation under conditions noted. Ratios of residual activity to initial activity are reported.

Relationship Between Stability and Activity in Evolved HRPs

HRP 2-13A10 used as template for the first round of error-prone PCR was isolated in previous experiments aimed at obtaining higher total activity in *S. cerevisiae* (Morawski et al., 2000). Stability was not considered during screening of these libraries and was allowed to drift. As a result, HRP 2-13A10 is less thermostable than wild-type (Figs. 1, 4). Combined screening for stability and activity should allow the discovery of any mutations that might increase stability without decreasing activity. Mutations improving both properties simultaneously, however, are rare (Giver et al., 1999). Screening a total of 11,300 colonies of the first libraries generated by error-prone PCR gave mutants with increased activity and the same stability as the parent. In the second generation, we found two mutants with increased stability, but their total activities both decreased. An exception to this trend is HRP 13A7 (Figs. 1, 2), which was isolated from the StEP recombination library. Here, a mutation introduced during earlier rounds of error-prone PCR reverted to wild-type and improved thermostability to the wild-type level at essentially no cost to activity (Fig. 4).

Expression of HRP Mutants in *S. cerevisiae* and *P. pastoris*

The total activities of thermostable HRP mutants 13A7, H2-10G5, and 13A7-N175S in *S. cerevisiae* are 20-, 10-, and 12-fold, respectively, greater than wild-type HRP. This represents an improvement of 40-fold in total activity compared to an earlier report of HRP expression in *S. cerevisiae* (Vlamiš-Gardikas et al., 1992). While sufficient for directed evolution, these levels are nonetheless low, and it is desirable to express the mutants in another host for purification and further characterization.

Wild-type HRP and mutants 13A7, H2-10G5, and 13A7-N175S were expressed in *P. pastoris* (Morawski et al., 2000) and purified from the supernatants. As observed previously, moving to *P. pastoris* increased total activity ~4–5-fold compared to *S. cerevisiae* (without optimization). Estimated from the total activities in the *P. pastoris* supernatant and the specific activities of purified protein samples (see below), HRP expression levels in *P. pastoris* are ~1.3 mg/l for wild-type, 5.5 mg/l for 13A7, 7.3 mg/l for H2-10G5, and 6.0 mg/l for 13A7-N175S.

The increase in the overall catalytic efficiency of the mutants is 2–3.9-fold for the purified mutants, compared to 10–20-fold for total activity in the *S. cerevisiae* supernatant, indicating that the HRP mutants are also expressed at higher levels than wild-type in *S. cerevisiae*. Comparing specific activity and total activity in *Pichia* cultures is not meaningful since the copy number of HRP genes in the *Pichia* genome may vary.

Stability of Purified Mutants

Thermostabilities of wild-type and mutant HRPs were investigated under different conditions of pH (pH 4.5, 7.0, and 8.0) and in the presence of 0.5% SDS, 8 M urea, and 3 M NaCl. In all cases the evolved mutants were more stable than wild-type HRP (Table I). Half-lives of inactivation measured at 55 and 60°C (pH 7.0) with the purified enzymes showed the same characteristics determined during screening unpurified enzymes in the *S. cerevisiae* supernatant. The half-life at 55°C increased from 3 min for wild-type to 16 min for H2-10G5 and 18 min for HRP 13A7-N175S. At 60°C, half-lives went from 3 min for wild-type to 7 min for H2-10G5 and 10 min for 13A7-N175S. Data for inactivation at 60°C (pH 7.0) are shown in Figure 5.

Sensitivity to H₂O₂ was determined after incubation of the purified enzyme samples in 1 mM H₂O₂ at 37°C, 50°C and 60°C in NaOAc buffer (pH 4.5). HRP H2-10G5 and

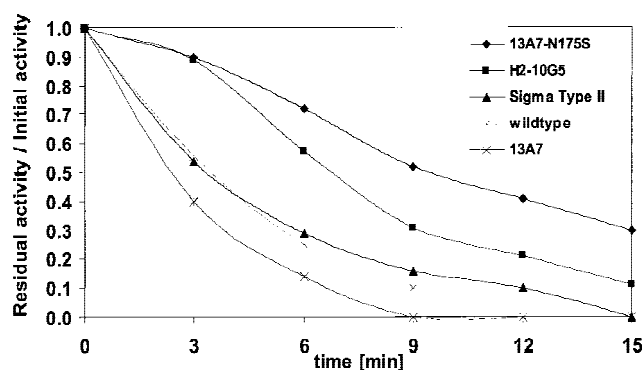


Figure 5. Time course of inactivation at 60°C of HRP-C Type II (Sigma), purified HRP wild-type, and variants after expression in *P. pastoris*. Samples were incubated in phosphate buffer (50 mM, pH 7.0) and aliquots were taken at different time points. Initial and residual activity were determined using ABTS as substrate.

13A7-N175S showed no increase in stability at 37°C, but were more resistant (1.5–2-fold) to inactivation by H₂O₂ at 50°C and 60°C (Table I). This shows that the improvement found under screening conditions (1 mM H₂O₂, 50°C) is solely due to thermal stabilization.

HRP from *P. pastoris* is more highly glycosylated (65%) than the native HRP Type II preparation obtained from Sigma (20%). The higher degree of glycosylation, however, does not influence the thermostability or activity of the wild-type enzyme (Table I).

Kinetic Constants and Specific Activities of Purified HRPs Expressed in *P. pastoris*

Kinetics for the HRP-catalyzed oxidation of ABTS and guaiacol are reported in Table II. The overall catalytic efficiency is represented by V_{\max}/K_m . V_{\max} was used because the molecular weight varies between 66 and 100 kDa (as judged from migration on SDS gels) due to glycosylation. (The mutations did not significantly affect this distribution.) The catalytic efficiency towards ABTS is higher than wild-type for all three mutants studied. HRP 13A7 showed the largest increase (3.9-fold). K_m values decreased slightly. The largest decrease, found for HRP 13A7-N175S, fully accounts for the increased catalytic efficiency of this mutant. Values for guaiacol show a different pattern (Table II). Here, mutant H2-10G5 showed the largest overall catalytic efficiency (2.9-fold increase with respect to wild-type), which results from an increase in V_{\max} .

K_m values for H₂O₂ were also determined (Table II). Interestingly, the K_m value for HRP 13A7-N175S decreased compared to wild-type. The difference in K_m for the preparation from Sigma and HRP-C wildtype is most likely due to presence of different isoforms in the purchased preparation.

Specific activities were determined for the substrates most frequently used in diagnostic applications (ELISA tests): tetramethylbenzidine (TMB), 5-aminosalicylic acid (5AS), *o*-phenyldiamine (OPD), and *o*-dianisidine (ODI) (Fig. 6). In general, the specific activities of the mutants are higher than wild-type. Figure 6 shows some interesting changes in substrate specificity as well. H2-10G5 and

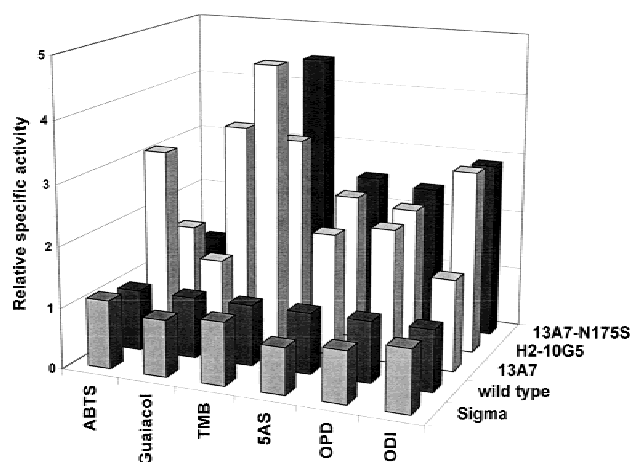


Figure 6. Specific activities of HRP-C Type II (Sigma), HRP wild-type, and variants expressed in *P. pastoris* towards different substrates. Activities are reported relative to activity of wild-type HRP. Substrates tested included 2,2'-azino-bis-(ethylbenzthiazoline-6-sulfonate) (ABTS), tetramethylbenzidine (TMB), guaiacol, 5-aminosalicylic acid (5AS), *o*-phenyldiamine (OPD), and *o*-dianisidine (ODI). Reported are the means of four separate experiments, with deviations from the mean of 5–10%.

13A7-N175S show no increase in activity towards ABTS, but are both more active towards the smaller aromatic substrates. The N175S substitution apparently influences the environment of the active site to promote reaction with smaller substrates over more bulky substrates like ABTS.

Mutations in Evolved HRPs

Starting parent HRP 2-3A10 already includes five amino acid substitutions (Fig. 3). Most (four) are located on the surface of the enzyme, as has been observed in many previous directed evolution studies. Random mutagenesis at low error-rates in fact favors the discovery of beneficial mutations at uncoupled, or structurally tolerant sites, many of which are located on the protein surface (Voigt et al., 2001).

HRP H1-8H10 isolated in the first round of random mutagenesis contains two new mutations, one leading to amino acid substitution N212D and a second, synonymous muta-

Table II. Kinetic parameters for HRP-C Type II (Sigma) and for HRP-C wild-type and variants expressed in *Pichia pastoris* and purified from the supernatant

Enzyme	ABTS			H ₂ O ₂	Guaiacol		
	K_m (mM)	V_{\max} (U/mg)	V_{\max}/K_m (U/mg/M)	K_m (μ M)	K_m (mM)	V_{\max} (U/mg)	V_{\max}/K_m (U/mg/M)
HRP Type II (Sigma)	0.53	9.8×10^2	1.85×10^6	190	1.5	1.0×10^2	6.9×10^4
HRP wild-type	0.68	8.7×10^2	1.3×10^6	93	1.5	1.1×10^2	7.4×10^4
HRP 13A7	0.53	27×10^2	5.1×10^6	210	1.2	1.5×10^2	13×10^4
HRP H2-10G5	0.49	14×10^2	2.8×10^6	99	1.7	3.7×10^2	21×10^4
HRP 13A7-N175S	0.36	9.8×10^2	2.7×10^6	51	1.2	1.3×10^2	10×10^4

Kinetics for ABTS oxidation were determined using 13–58 ng/ml of protein, 3 mM H₂O₂, and 0–1.8 mM ABTS in NaOAc buffer (50 mM, pH 4.5). Kinetics for H₂O₂ reduction were determined with 3–11 ng of purified enzyme, 3 mM ABTS, and 0–0.1 mM H₂O₂. Kinetics for guaiacol oxidation were performed with 32.5–116 ng/ml of protein, 3 mM H₂O₂, 0–5 mM guaiacol in 50 mM sodium phosphate buffer (pH 7.0). Protein concentration was determined after Bradford with BSA as standard. Reported are the means of three separate experiments with deviations from the mean of 8–10%.

tion at position A85, GCC → GCT. N212D is located in the loop between helix F' and strand 2 (N212 is not a glycosylation site) (Fig. 7). HRP H1-6E1 contains one new mutation leading to substitution of lysine 232 by glutamic acid at the beginning of helix G.

In mutant HRP 13A7 created by StEP recombination, Q223 reverted to the wild-type leucine and increased thermostability. Located near Ca²⁺ ligands D222 and T225 (Gajhede et al., 1997), this mutation may exert its influence on thermal stability by affecting Ca²⁺ binding (Hascke and Friedhoff, 1978). The N175S substitution is responsible for the increased thermostability of H2-10G5, 13A7-N175S, and wild-type-N175S. Position 175 is in strand 1 (Fig. 7) and is near the active site of the enzyme (N175 is not a glycosylation site). The most stabilizing mutation at this position is a change from Asn to Ser, as shown by saturation mutagenesis. Investigation of the structure using SwissPdB Viewer indicates that N175S introduces a new hydrogen bond. In wild-type HRP, N175 forms two hydrogen bonds (2.83 and 2.79 Å) with V219, located in strand 2. Changing Asn to Ser introduces a third hydrogen bond (3.3 Å) to V219 (hydrogen bonds are between Val219N and Ser175O, and between Val219O and Ser175N and Ser175OH), which might stabilize the environment around the heme.

Recently, Iffland et al. (2000) identified several mutations by directed evolution that increase the total and specific activities of cytochrome c peroxidase towards guaiacol. One of the mutations repeatedly found in their isolated mutants was T180A. This position corresponds to N175 in HRP. T180A increases the affinity of the apo form of CCP for heme. Unfortunately, the thermostabilities of those mutants were not reported.

Studies carried out on *iso*-1-cytochrome c, a heme protein that catalyzes peroxidase-like reactions in vitro (Vazques-

Duhalt, 1999), have shown that a mutation of N52 located close to the active site to isoleucine strengthens global protein stability by increasing the local stability of the heme crevice. This is achieved by decreasing the cavity size around the heme and leads to lower reactivity (Schejter et al., 1994). N52I also stabilizes cytochrome c heme against degradation by H₂O₂ (Villegas et al., 2000). The phenotype of this N52I *iso*-1-cytochrome c mutant resembles our N175S HRP mutant and suggests a similar role of the Asn residues in the two enzymes.

An alternative explanation for the effect of this mutation is that replacement of Asn by Ser protects the enzyme against spontaneous, nonenzymatic deamidation. Hydrolytic deamidation of labile Asn and Gln residues can occur under acidic, neutral, or alkaline conditions (Volkin et al., 1995), and the rate depends on the primary sequence as well as tertiary structure. Lys occurs disproportionately often on the amino acid side of a labile Asn (Wright, 1991). A Lys residue precedes the mutated N175. Deamidation usually occurs, however, during incubation for a longer time period (several hours up to days) than the brief incubations (10 min) investigated here. Our preferred speculation as to the role of N175S is that it stabilizes the protein by stabilizing the heme cavity.

Comparison to Other Peroxidases

In an earlier directed evolution study, Cherry et al. (1999) dramatically increased the thermal and oxidative stability of the *Coprinus cinereus* (CiP) heme peroxidase, using a combination of site-directed mutagenesis, random mutagenesis, and in vivo shuffling. Comparable levels of improvement were not achieved in the current study on HRP. One explanation may be that HRP is already thermostable compared to CiP: CiP is inactivated after only 5 min at 40°C (Cherry et al., 1999), while HRP retains 100% activity at this temperature (data not shown). Thus, CiP may have many more opportunities for further stabilization than does HRP. Cherry et al. also screened a much larger number of mutants (140,000 vs. ~10,000).

Naturally thermostable peroxidases have been described and include soybean peroxidase (McElDoon and Dordick, 1996), extracellular peroxidases from *Streptomyces thermoviolaceus* (Iqbal et al., 1994), and an intracellular peroxidase from *Bacillus sphaericus* (Apitz and van Pee, 2000). Relatively little is known, however, of the reactivities and substrates of these enzymes compared to the widely used, but less thermostable, horseradish peroxidase.

The use of HRP-labeled primary or secondary antibodies for different hybridization reactions (Northern, Southern, or Western Blot and ELISA) has increased in the last decade mostly due to the development of sensitive detection methods for HRP through the introduction of new substrates (Conyers and Kidwell, 1991; Sudhaharan and Reddy, 1999). Applying the improved HRP variant described here, or using similar methods to increase activity towards these substrates, could decrease the detection limits even further.

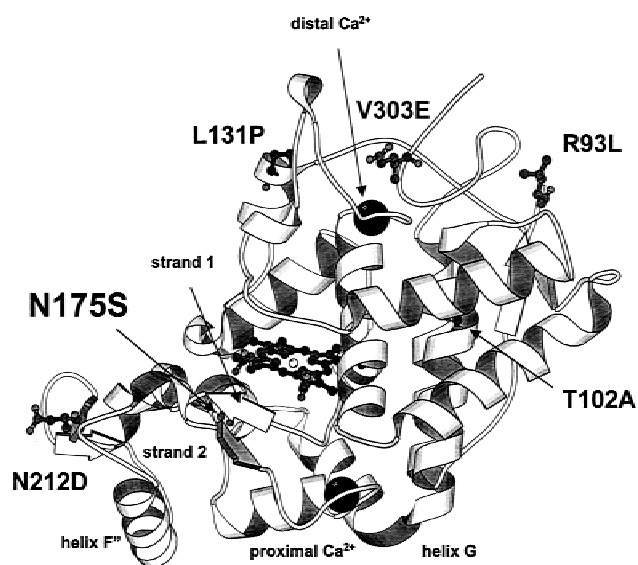


Figure 7. Amino acid substitutions found in HRP 13A7-N175S. Mutation N175S located in close proximity to the heme is found to be responsible for an increase in thermal stability. The two Ca²⁺ ions are shown as black balls.

The improved HRP variant may also be useful for bioremediation, polymer production, or in sensor applications, where increased stability leads to reduced catalyst costs.

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